Liao *et al. Zoological Studies* 2013, **52**:21
http://www.zoologicalstudies.com/content/52/1/21

Zoological Studies
a SpringerOpen Journal

RESEARCH Open Access

Reproductive behavior of the male rose bitterling *Rhodeus ocellatus* as influenced by the operational sex ratio

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Abstract

Background: Game theory predicts that as the operational sex ratio (OSR; the ratio of sexually active males to fertilizable females) increases, reproductive males may adopt alternative reproductive behaviors. Empirical studies indicated that alternative behaviors may differ for different species. In the present study, we investigated the influence of different male-biased OSRs on male reproductive behaviors shown by a freshwater fish, the rose bitterling *Rhodeus ocellatus*.

Results: Six levels of the OSR (1:1, 2:1, 3:1, 5:1, 7:1, and 9:1) were established, and male leading, aggression, and preoviposition ejaculation behaviors were recorded. Our experiments showed that at low OSRs, the leading behavior of male rose bitterling increased with an increasing OSR, reaching a maximum at an OSR of 3:1, and then decreased as the OSR further increased. In contrast, aggression and preoviposition ejaculation behavior continually increased with an increasing OSR. Notably, males possibly lost their territorial position to other males and became sneakers when facing more rivals.

Conclusions: These observations suggest that at lower OSRs, male rose bitterling mainly rely on leading behavior, while at higher OSRs, they increasingly adopt aggression and preoviposition ejaculation. Therefore, for the rose bitterling, (1) decreased leading behavior and (2) increased aggression and preoviposition ejaculation are two alternative responses in the face of an increasing OSR, which is a more straightforward strategy for facing an increased risk of sperm competition than that adopted by its congener, the European bitterling.

Keywords: Game theory; Alternative behaviors; Male mating behavior; Rose bitterling

Background

Sexual selection was suggested to be an important evolutionary driver about which extensive studies have been conducted (e.g., Andersson 1994). While sexual selection may be very complex and influenced by many factors, the operational sex ratio (OSR; the ratio of sexually active males to fertilizable females) was suggested to be closely related to the intensity of sexual selection and recently received great attention (Reichard et al. 2004a; Klug et al. 2008; Dur et al. 2012). The OSR may influence the distribution and variance in reproductive success among individuals in a population, and it may be a strong driving force in the evolution and maintenance of sexual characteristics (Weir et al. 2011).

Classical sexual selection theory predicts that as the OSR increases, males compete more intensely for females and/or for the resources that females need. This theory well explains the evolution of secondary sexual characteristics such as male ornamentation and elaborate courtship displays in many species (Clutton-Brock and Vincent 1991; Andersson 1994; Reynolds 1996). However, concepts of economic defensibility of resources (Emlen and Oring 1977) and game theory models (Parker et al. 1996) emphasize that when resources cease to become economically defendable and territorial males become unable to capitalize on the environmental potential for polygamy, males may abandon territoriality altogether (Grant et al. 1995) or adopt an alternative reproductive behavior with direct sperm competition to

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gain access to females without courtship (Gross 1996; Taborsky 1998).

Weir et al. (2010) investigated aggressive behavior in relation to the OSR within alternative phenotypes (large anadromous males and small ‘sneaker’ mature male parr) of Atlantic salmon *Salmo salar* but found that they exhibited different responses with an increase in the OSR. Therefore, they concluded that there was considerable variability in competitive behaviors among different species. Mills and Reynolds (2003) also suggested that correlations of the OSR with resource competition and variance in mating success depend on details of the biology of a particular species. Different alternative behaviors when facing different OSRs by different species still need to be tested.

Bitterlings are a group of freshwater fishes that belong to the subfamily Acheilognathinae (family Cyprinidae), which have a unique spawning symbiosis with freshwater mussels. During the spawning season, males develop bright nuptial coloration and defend territories around mussels. Female bitterlings develop long ovipositors that they use to place their eggs onto the gills of a mussel through the mussels’ exhalant siphon. Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel, so that water filtered by the mussel carries the sperm to the eggs. Bitterlings display remarkable morphological, physiological, and behavioral adaptations for using mussels as spawning sites, and they represent a valuable model in behavioral, population, and evolutionary ecology (Smith et al. 2004).

By establishing two different male densities, Mills and Reynolds (2003) compared different reproductive behaviors of the European bitterling *Rhodeus sericeus*. They found that at a higher male population density, large males chased other males, guarded territory, courted female much less often, and significantly reduced their sperm release.

Candolin and Reynolds (2002) compared the reproductive behaviors of the European bitterling at densities of 1, 2, 4, or 6 males and found that the rate of ejaculation initially increased and then decreased with an increase in male density. They also found that a negative relationship occurred between changes in aggression and changes in the ejaculation rate of the dominant male. They suggested that a decreased ejaculation rate and increased aggression are alternative responses to increased risk of sperm competition.

Notably, Reichard et al. (2004a) manipulated male densities at the local and population levels and found that more male-male interactions led to an interruption of spawning, with the result of decreased egg production by the entire population at high male densities. However, they observed a decline in male aggression at the highest male density, causing stabilization in egg production. This illustrates that male mating behaviors can also have important population and demographic consequences (Reichard et al. 2004a; Konečná et al. 2010).

Through extensive field observations of European bitterling reproduction, Smith et al. (2003) found that male competition intensity was largely in accordance with predictions of the game theory model: both ejaculate expenditure and the territorial male aggression rate initially increased and then decreased with an increase in male density.

The rose bitterling *Rhodeus ocellatus* is a congener of the European bitterling, which is distributed in East Asia, and exhibits similar reproductive behaviors. Some works were conducted on the reproductive behaviors of the rose bitterling (Kanoh 1996, 2000; Casalini et al. 2009, 2010; Agbali et al. 2010). The influence of different male-biased OSRs on male reproductive behaviors of the rose bitterling has not been investigated yet. In the present study, by setting up a series of experiments, we studied the reproductive behavioral responses of male rose bitterlings to an increasing OSR. Specifically, we tested whether male rose bitterlings would employ similar alternative reproductive behaviors as the European bitterling does and what the potential evolutionary consequences would be.

Methods

Field collection

Bitterlings for all experiments were collected from Bao’an Lake (114°23′E, 30°15′N; at 20 m in elevation) of the Yangtze River Basin in Hubei Province, China. Mussels of the species *Unio douglasiae* were collected in Poyang Lake (Yangtze River Basin) using a long-handled dip net. This mussel species was used in the present study because it is the preferred host species of the bitterling (Reichard et al. 2007b). Captured fish and mussels were transported to the Institute of Hydrobiology, Wuhan, China and kept in stock aquaria with fresh water in the laboratory. The bitterlings were separated by sex under a natural light regime and fed with frozen chironomid larvae and commercial fish flakes. The present study was conducted in accordance with Chinese legal requirements. Experimental procedures were approved by the Hubei Province Association for Laboratory Animal Sciences.

Reproductive behaviors

Experiments were conducted in a tank measuring 50 (length) × 45 (width) × 40 (depth) cm with a 5-cm-deep sand substrate. The mean water temperature during the experiments was 22°C. The OSR of rose bitterlings in the breeding period is typically male biased; Kanoh (2000) reported that OSRs varied 2:1 to 4.9:1 in a small pond. Therefore, the OSRs were set to 1:1, 2:1, 3:1, 5:1, 7:1, and 9:1. Before the experiment, a mussel was placed in a sand-filled plastic cup at the center of the tank, which was covered with an upturned perforated plastic cup to prevent females spawning in them, but all bitterlings were able to see and smell the mussel. Then, a single male bitterling was caught from the stock aquaria and gently released in the tank. After 30 min, the male had established his territory around the mussel, and the cup was removed; additional males were added to the tank as potential rivals. Finally, a female with a long ovipositor was selected from the stock aquaria and added to the test tank after introducing the males. All behaviors of the territorial male bitterling were recorded for 30 min, regardless of whether spawning occurred or not. We tracked the territorial male through repeatedly watching videos. Totally, we had 12 replicates for each OSR treatment with different individual fish (72 mature females and 262 males were used in total) and mussels (72 mussels), producing 72 trials in total.

The recording behaviors were derived from Wiepkema (1961) and Smith et al. (2004), and frequency estimates were made for the following behaviors: (1) leading, in which a territorial male guided a female towards a mussel while courting; (2) aggression, in which a territorial male moved towards other males, including fin spreading, parallel swimming, jerking, and head-butting; and (3) preoviposition ejaculation, in which sperm release was directed by the territorial male, sweeping forward and down quickly over the inhalant siphon of the mussel; it was sometimes visible as a grayish cloud.

At a high density and OSR, territorial males were possibly displaced by other rivals during courtship, and the displacement was defined as a non-territorial male chasing away the territorial male and occupying the mussel to become a new territorial male. All behaviors were recorded for the first territorial male for 30 min, regardless of whether territorial displacement occurred or not. After completing the observation of behaviors, the fish were removed and measured (standard length (SL), from the tip of the snout to the origin of the base of the tail fin), and these male and female bitterling and mussels were not used again. The average SL of territorial males was 47.14 ± 1.14 mm and that of sneakers was 37.03 ± 0.89 mm.

The breeding season of the rose bitterling begins in April to September, and the peak appears during May to June (Shen 2000). We conducted our research separately from 14 July to 5 September 2006 for OSRs of 1:1 and 2:1, 11 to 14 May 2007 for OSRs of 3:1 and 5:1, 14 May to 13 July 2008 for the OSR of 7:1; and 17 May to 16 June 2009 for the OSR of 9:1. All experiments were carried out by the same person.

Video images and data analysis

The 30-min video was divided into three 10-min sections for behavioral observations. There were no significant differences found in the same behavior among these three 10-min sections. The mean value of the three 10-min sections was used as the input data so that the numbers of behaviors were more stable. A one-way analysis of variance (ANOVA) was used to test for differences in territorial males' behaviors of leading, aggression, and preoviposition ejaculation among the six densities and OSRs. Tukey's *post hoc* tests were used to reveal specific differences among independent variables. The proportion of the first territorial male being displaced was obtained by the number of times in the experiments that the first territorial male was displaced divided by 12 replicates. A Pearson correlation was used to test the correlation between the OSR and territorial male displacement. Statistical analyses were performed using SPSS version 13.0 (SPSS, Chicago, IL, USA).

Results

Territorial male reproductive behaviors

As the male density and male-biased OSR increased, the leading frequency of territorial males showed a distinctive pattern. There was a low level of leading when no rival was present, but it increased to a maximum when two rivals were present. Further increases in the numbers of rivals resulted in a decline in the leading frequency (one-way ANOVA $F_{5,66} = 6.181$, $p < 0.001$; Figure 1A). There were no significant pair-wise distinctions in the leading behavior between OSRs of 1:1 and 5:1, 1:1 and 7:1, and 1:1 and 9:1 (Tukey's *post hoc* tests, all $p > 0.05$). However, there were significant differences between OSRs of 1:1 and 2:1, and 1:1 and 3:1 (Tukey's *post hoc* tests, both $p < 0.001$). Territorial males exhibited significantly more aggression before spawning as the male density and male-biased OSR increased (one-way ANOVA $F_{5,66} = 13.826$, $p < 0.001$; Figure 1B). Significant pair-wise distinctions were detected between OSRs of 1:1 and 5:1, 1:1 and 7:1, 1:1 and 9:1, and 2:1 and 9:1 (Tukey's *post hoc* tests, all $p < 0.05$). The mean number of sperm releases prior to spawning (preoviposition ejaculation) increased with an increasing number of competing rivals (one-way ANOVA $F_{5,66} = 3.730$, $p = 0.003$; Figure 1C). The only significant difference found in the pair-wise comparisons was between OSRs of 1:1 and 9:1 (Tukey's *post hoc* test, $p = 0.002$).

Territorial displacement

A notable phenomenon was observed in our experiment. Territorial males could defend their territories when faced with a single rival, but they possibly lost their territorial position to other males and become sneakers when facing a greater number of rivals. At an OSR of 1:1, the territorial male was never replaced by another male; therefore, it was not included in our ANOVA. However, it was found that with an increasing number

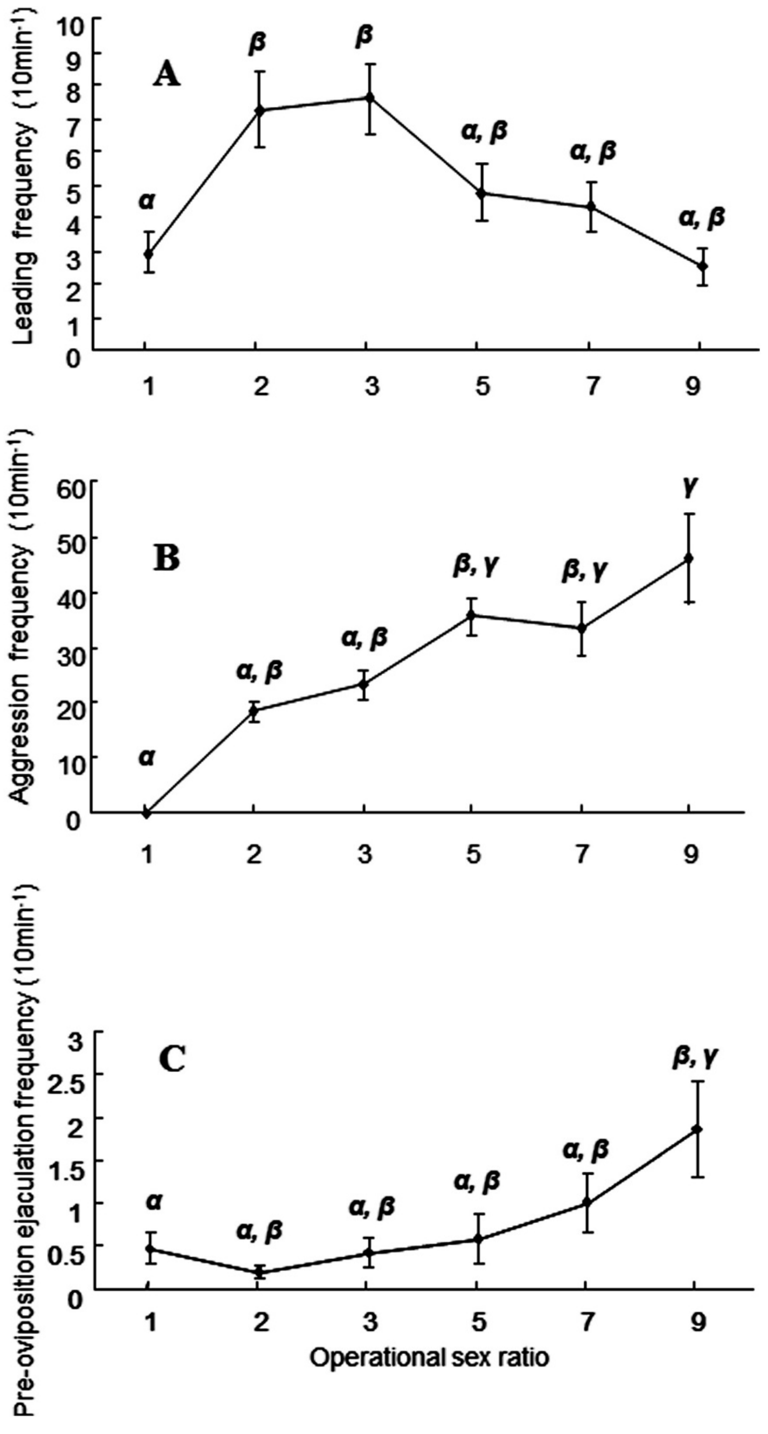

Figure 1 Territorial male reproductive behaviors in different operational sex ratios. (A) mean (±SE) frequency of leading, **(B)** mean (±SE) frequency of aggression, and **(C)** mean (±SE) frequency of preoviposition ejaculation. The significance ($p < 0.05$) of *post hoc* pair-wise comparisons is indicated by lowercase Greek letters (α, β, and γ) above the error bars; sharing any letter among different OSR treatments indicates that there was no significant difference.

of competing rivals, the proportion of territorial male displacement increased (one-way ANOVA $F_{4,55} = 5.230$, $p = 0.001$; Figure 2). There were significant pair-wise differences between OSRs of 2:1 and 7:1, and 2:1 and 9:1 (Tukey's *post hoc* test, $p < 0.05$). The proportion of territorial displacement increased to >50% when six or eight

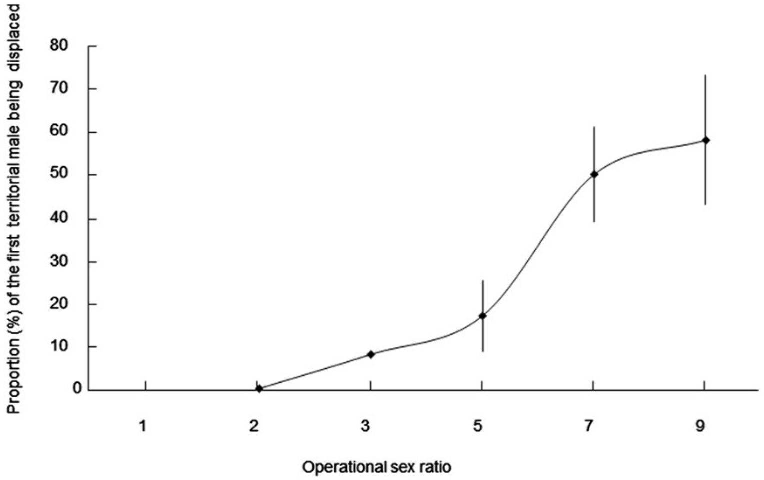

Figure 2 **Mean (±SE) proportion (%) of territorial displacement in different operational sex ratios.**

rivals were present. There was a significant correlation between the OSR and territorial displacement (Pearson correlation $r = 0.546$, $p < 0.001$).

Discussion

Alternative reproductive behaviors of male rose bitterlings

Our experiments showed that the reproductive behaviors of male rose bitterlings were influenced by the OSR. At low OSRs, the leading behavior increased with increasing OSRs, reaching a maximum at an OSR of 3:1, and then decreased as the OSR further increased, which was just as the game theory predicts (Parker et al. 1996). However, aggressive behavior continued to increase with an increasing OSR, corroborating the results of a previous study of this species (Kanoh 2000), which differs from the European bitterling (Reichard et al. 2004a).

In the present study, the preoviposition ejaculation by male rose bitterlings also showed a continually increasing pattern as the OSR increased. In contrast, Kanoh (1996) found that the territorial male rose bitterling never performed preoviposition when there were sneaker males present. In fact, our results demonstrated that only at OSRs of 7:1 and 9:1 did the frequency of preoviposition ejaculation exceed once per 10 min, but the OSR in Kanoh's (1996) experiment was only 3.5:1, so there is no contradiction between these two studies. Preoviposition ejaculation may be more likely to appear at greater male-biased OSRs for the rose bitterling. Therefore, for this species, decreased leading vs. increased aggression and preoviposition ejaculation could be alternative responses to an increasing OSR.

For the European bitterling, Mills and Reynolds (2003) found that with high male population densities, large males chased other males, guarded territory, courted females much less often, and significantly reduced their sperm release. Candolin and Reynolds (2002) found that the rate of ejaculation showed an initial increase, then a decreasing pattern with an increase in male density, and a negative relationship occurred between changes in aggression and changes in the ejaculation rate of the dominant male. Smith et al. (2003) found that both ejaculate expenditure and the territorial male aggression rate exhibited initial increases and then decreasing patterns with increasing male density.

Although the results of the European bitterling were a little ambiguous, Candolin and Reynolds (2002) suggested that in the European bitterling, a decreased ejaculation rate and increased aggression were alternative responses to an increased OSR, and this was recently confirmed by Řežucha et al. (2012).

Compared to the European bitterling, the male rose bittterling showed different alternative behaviors in the face of an increasing OSR. At lower OSRs, males mainly relied on leading behavior, while at higher OSRs, they increasingly adopted aggression and preoviposition ejaculation. We considered that at lower OSRs, courtship such as leading would work well; at higher OSRs, increasing aggression and preoviposition ejaculation would be more effective for sperm competition. Previous studies on this species also showed that during courtship at a low density, males showed more courting actions than at a high density (Warner and Hoffman 1980; De Boer 1981; Kanoh 2000). Therefore, the rose bitterling adopted a more straightforward strategy when facing an increased risk of sperm competition than the European bitterling.

The OSR can influence the structure of the breeding system independently or concomitantly with the density (Kodric-Brown 1988). In the case of the rose bitterling, experimental studies confirmed the role of a high male density in increasing male aggressive behavior (Kanoh 2000).

However, Casalini et al. (2010) found that the density did not influence overall male aggressive behaviors but had a significant effect on the male courtship rate. In the present study, the male density varied with changes in the OSR. We think that both male density and the OSR might together influence the reproductive behaviors of rose bitterlings.

In this study, there was a potential shortcoming in the experiment design. All separate sex ratios were tested sequentially rather than in a random order over several years. We could not statistically test for a year effect on the reproductive behaviors.

Possible evolutionary consequences of high OSRs

Generally, in the wild, the overall sex ratio of bitterlings is 1:1 (Zhang 2005). Since females spawn every 2 ~ 3 days and males can court all the time, the OSR is typically male-biased. Therefore, different settings of the sex ratio in our experiments have important consequences for the ecology and natural history of the species (Konečná et al. 2010). We suggest that a prevailing male-biased OSR could have some evolutionary consequences.

In the present study, we found that when faced with an increasing OSR, male rose bitterlings initially showed an increase and then a decrease in the courtship intensity such as leading, but a continually increasing ejaculation frequency and aggressive behavior. Thus, over a long time, at lower OSRs, sexual selection could lead to the evolution of characteristics related to courtship, such as a large body size and bright color in males; at higher OSRs, sexual selection would favor the evolution of characteristics related to aggression, possibly larger body sizes in males.

Using behavioral and genetic data, Reichard et al. (2004b) investigated how male density affected the reproductive success of territoriality and sneaking in the European bitterling. They found that territorial mating was almost 17 times more successful than sneaking at the lowest male density treatment and still two to three times more successful at intermediate densities. However, both behaviors conferred the same fitness payoff at the highest male density. They considered that preoviposition sperm loading was the best predictor of male reproductive success rather than aggression, body size, or post-oviposition ejaculation. This indicates that at extremely high male-biased OSRs, characteristics related to sneaking would be favored, and the evolution of aggression would be limited (Reichard et al. 2007a) or the evolution of a larger male body size would be constrained (Reichard et al. 2009). We think that a paternity analysis of different behaviors at different OSRs in the rose bitterling would reveal interesting results on the evolutionary consequences of different OSRs.

Conclusions

In conclusion, the reproductive behaviors of male rose bitterling are influenced by male-biased OSR. Decreased leading behavior and increased aggression and preoviposition ejaculation are two alternative responses in the face of an increasing OSR, which is a more straightforward strategy for facing an increased risk of sperm competition than that adopted by its congener, the European bitterling. This study provides strong evidence that male-biased OSR has an important effect on male reproductive behaviors. It has very important significance for better understanding of animal mating strategies.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CL carried out the behavioral experiments and drafted the manuscript. DY participated in the data analysis. YC participated in the discussion. HL participated in the design of the study and provided financial support. All authors read and approved the final manuscript.

Acknowledgements

We are grateful to Rowena Spence for her useful comments on the manuscript. We would also like to thank Yu Zeng, Yuyu Xiong, Jinming Wu, and Shaorong Yang for their help in collecting the samples and to the two anonymous reviewers for their valuable suggestions. Funding was provided by the National Natural Science Foundation of China (grant no. 30970443).

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Received: 18 November 2012 Accepted: 16 July 2013
Published: 30 September 2013

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doi:10.1186/1810-522X-52-21
Cite this article as: Liao *et al.*: Reproductive behavior of the male rose bitterling *Rhodeus ocellatus* as influenced by the operational sex ratio. *Zoological Studies* 2013 **52**:21.